

Original Research Article

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Markers of HCV-Related HCC in Egyptian Patients

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ABSTRACT

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At least two thirds of HCV infected cases would probably develop chronic HCV infections, and are thus at risk of developing liver cirrhosis and HCC. We investigated the sequence of hepatitis C virus RNA NS5A region in HCV-related HCC patients as well as in chronic HCV patients to highlight its potential pathogenic role, and investigated the usefulness of TGF-β1 and ICAM-1 as serological biomarkers in the diagnosis of HCV-related HCC. The results in this study indicated that 60% of group I (HCV-related HCC) and 23.3% of group II (chronic HCV) patients harbored a wild-type sequence of NS5A region with a significant difference between both groups. Moreover, there is significant increase in mean sequence identities in group I, compared to group II ($p < 0.001$) and significant increase in mean number of mutations in NS5A, and ISDR regions in group II ($p < 0.001$) compared to group I, and serum TGF-β1 and ICAM-1 levels in group I patients were significantly higher than those in group II patients ($p < 0.001$). Conclusion: The wild type NS5A, particularly that of ISDR, is a significant marker for the development of HCV-related HCC. In addition, TGF-β1 and ICAM-1 are potential effective serological markers for the diagnosis of patients with HCV-related HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer, and the second most common digestive system malignant tumor worldwide, accounting for 5% of all cancers (1,2). Hepatitis C virus (HCV) is considered to be the second most common cause of HCC, accounting for 25% of HCC cases (3). In fact, HCV infections is a global serious health threat, as 3.5 million new HCV infected cases and 350000 deaths from HCV

related diseases are reported annually (4). More than two thirds of HCV infected cases would probably develop chronic HCV infections, and are thus at risk of developing liver cirrhosis, liver cell failure and HCC (5). Despite the decrease in the prevalence of HCV in developed countries, the developing countries, including Egypt, still exhibit increasing prevalence rates of HCV infections, which is recently reported to be

about 10-14% of Egypt's population (3,6,7).

A better understanding of the molecular pathogenesis of HCV would indeed help to establish novel strategies for the prevention and treatment of HCV related diseases, including HCC. HCV is a small enveloped single stranded hepatotropic RNA virus that belongs to Flaviviridae family, with its 9.6 kb genomic RNA consisting of 3 main regions: The highly conserved 5'UTR, an open reading frame (ORF) that encompasses about 9000 nucleotides, and the 3' UTR regions. The HCV ORF encodes a 3000 long polypeptide chain which is then cleaved into 3 structural and 7 non structural proteins, namely core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (8,9,10). It is known that the non-structural 5A region (NS5A) plays an important role in promoting both the viral replication and assembly of the viral particles. It was also reported that NS5A could possibly help the virus to escape the host immune response and thus aids the virus pathogenesis and spread. Moreover, previous studies on transgenic mice demonstrated that NS5A induced genome instability, dysregulated cellular growth and signal transduction and suggested that it might have a important role in HCC pathogenesis (11,12). All these findings make NS5A a critical target for antiviral drugs, but as it is known that different HCV genotypes exhibit different responses to HCV antiviral therapies, it is important to clarify the potential pathogenic role of NS5A in Egyptian population where genotype 4 is the most prevalent genotype (9).

Great efforts have been made to identify new serological biomarkers for diagnosis of HCC. It was reported that induction of chronic inflammation with increased expression of TH2 cytokines and adhesion molecules creates a neoplastic favoring

microenvironment (13,14). Overexpression of TGF- β 1 was reported in a number of cancers, including HCC. Though TGF- β 1 is known as a tumor suppressor that suppresses cell cycle progression in early G1 phase, misregulation of TGF- β 1 signaling promotes tumor growth, cancer cell dissemination and metastasis (5). It was also reported that HCC cells secrete increased levels of ICAM-1, that would facilitate the adhesion and movement of cancer cells and thus promote tumor development (14,15).

The present work aimed to investigate the sequence of hepatitis C virus RNA NS5A in patients with chronic hepatitis C and in patients with HCV-related HCC to highlight its potential pathogenic role, and to investigate the usefulness of biomarkers as TGF- β 1 and ICAM-1 in the diagnosis of HCV related-HCC.

Materials and Methods

This study included 30 Egyptian patients with HCV-related HCC (group I), and 30 Egyptian chronic HCV patients (group II), who attended the clinic of Oncology unit-Nasr City Insurance Hospital and the clinics of Tropical Medicine and Internal Medicine-Ain Shams University Hospitals. All group I patients were diagnosed as having HCV-related HCC by quantitative PCR to detect HCV-RNA, AFP levels, combination of ultrasonography (US) and computed tomography (CT), and all of them had no evidence of extrahepatic metastasis. Meanwhile, all group II patients were chosen for the study after full laboratory data assessment including liver function tests, HCV-RNA detection by quantitative PCR, and genotyping, with no evidence of HCC development by AFP, US or CT. All the patients included in the present study (both group I and II) were HCV 4-a subtype which is the most prevalence subtype in

Egypt and were negative for hepatitis B surface antigen (HBsAg).

Informed consent was obtained from all participant individuals. The study was conducted in accordance with the stipulations of the local ethical and scientific committees of Ain Shams University and the procedures respected the ethical standards in Helsinki declaration of 1964. Samples were collected from all patients and plasma and serum were stored at -70°C until they were used.

All patients were subjected to:

1. Sequencing of the HCV-NS5A region (codons 2069-2265) by automated DNA sequencer (ALF express Autoread), using sequencing kit, in conjunction with ALF ExpressTM dATP labeling mix supplied by Amersham Pharmacia Biotech, Sweden.
2. Serum TGF- β 1 assay by commercially available Quantikine R&D Human TGF- β 1 ELISA Kit (R&D Systems, Minneapolis, MN, USA).
3. Serum ICAM-1 assay by commercially available Quantikine R&D Human ICAM-1 ELISA Kit (R&D Systems, Minneapolis, MN, USA).

Amplification of HCV NS5A by RT-PCR and Sequence Analysis

RNA extraction was done using QIA amp viral RNA Mini kit (QIAGEN Group), according to the manufacturer's instructions, followed by RNA reverse transcription and amplification using QIAGEN one step RT-PCR kit technology, USA. Primers used for amplification of NS5A region were primer A (forward primer): CCGTACCTT GGAAGGGGTAG, and primer B (reverse primer): ACCGAGACTT CCCTGTCATC. The reverse transcription and amplification

were done according to the following program: 50°C for 30 minutes, 95°C for 15 minutes, followed by 3 steps amplification cycle consisting of: 94°C for 1 min, 68°C for 1 min, 72°C for 1 min, which is repeated for 40 cycles, followed by final extension at 72°C for 10 min.

DNA sequence analysis for HCV NS5A region was done using ALF express Autoread sequencing kit, in conjunction with ALF ExpressTM dATP labeling mix supplied by Amersham Pharmacia Biotech, Sweden. This process involved denaturation of the amplified DNA followed by annealing of denaturated DNA with the primers (forward primer: CCGTACCT TGG AAGGGGTAG), and (reverse primer: ACCGAGACTTCCCTGTCATC). The annealing reaction was preheated at 65°C for 5 minutes, then placed at 37°C for 10 minutes and at room temperature for 10 minutes. This was followed by addition of the labeling mix and termination reactions.

The resulting nucleotides sequencing of amplified NS5A region were translated into individual amino acid using DNA MAN program (gene bank). Then the amino acid sequence alignment relative to establish wild type NS5A protein region of HCV-genotype 4-a sequence data base from gene bank to deduce the amino acid changes.

Statistical Analysis

Statistical analysis performed using SPSS V 15.0. Results were expressed as means \pm SD, percentages, and ranges. Comparisons of quantitative and qualitative variables were conducted between groups using the student-t test and Chi-square tests and - Fisher's exact test respectively. A p-value of ≤ 0.05 was considered to be statistically significant while a $p \leq 0.001$ was considered highly significant.

Logistic regression analysis was performed to determine the significant effect of the different variables on the diagnosis of chronic hepatitis C and HCV related HCC.

An operator characteristic (ROC) curve was constructed to establish clinically relevant cut off values for TGF- β 1 and ICAM-1.

Results and Discussion

The mean age of patients involved in this study was 56.23 ± 7.4 years, while the mean age for group I and II was 57.30 ± 8.1 and 55.2 ± 6.7 years respectively with no significant difference between both groups ($t=1.11$, $p=0.28$). There was male predominance in both groups, with group I including 3 females and 27 males and group II including 6 females and 24 males.

The mean HCV- RNA viral load was 269433 ± 207863 and 294597 ± 229284 IU/ml for group I and group II patients respectively, with no statistically significant difference between both groups ($p=0.575$).

Demographic and descriptive laboratory data of group I and group II patients are described in table (1)

Results of NS5A Sequencing

The results in this study indicated that 18 patients (60%) out of 30 patients of HCV-related HCC group (group I) harbored a wild-type (wt) sequence of NS5A region with a sequence identities (100%), while the other 12 patients (40%) harbored mutations that ranged from 2 - 6 with mean 4.5 ± 1.38 mutations/case and mean sequence identities 91.5 ± 1.31 %. On the other hand it was found that 23/30 (76.7%) of non HCC chronic HCV patients (group II) harbored mutations that ranged from 5 - 17 with a mean 9.5 ± 3.01 mutations/case and mean sequence identities 89.1 ± 3.82 %.

Comparative statistics between group I and group II revealed a significant higher frequency of mutations in the NS5A region in group II patients ($X^2=8.2971$, $p=0.003$). Also there was highly significant increase in mean sequence identities in group I compared to group II ($p<0.001$), and highly significant increase in mean number of mutations in group II ($p<0.001$) compared to group I (table 2).

Interferon Sensitive Determining Region (ISDR) Mutations (Codons 2209-2248)

10 out of 30 group I patients versus 23 out of 30 group II patients had ISDR mutations, with a highly significant difference between both groups ($X^2=11.381$, $p<0.001$). The mean number of mutations/case in the ISDR sequence was lower in group I patients than in group II patients with a highly significant difference between both groups ($p<0.001$)(table 2 and 3). The mutations in the ISDR in group II patients ranged from 1-7, while none of group I patients had more than 1 ISDR mutation.

Figure (1) shows schematic representation of the amino acids sequence of the amplified NS5A region for patients involved in this study

Comparative statistics between group I and group II patients regarding the studied parameters are shown in table (2), while comparative statistics between group I and group II mutation positive patients regarding the studied parameters are shown in table (3).

Regarding the frequency of mutations in the NS5A region, it was found that mutations at codons 2260 & 2130 were the most frequent occurring in 21/23 group II and 8/12 group I patients who harbored NS5A mutations. Meanwhile, mutation at codon 2212 was the most frequent mutation in ISDR region

occurring in 19/23 group II patients and in 9/12 group I patients who harbored NS5A mutations.

Results of TGF-β1 and ICAM-1

The present study results showed that the mean TGF-β1 and ICAM-1 levels in the group I patients were significantly higher than those in group II patients (both $p < 0.001$). The serum levels of TGF-β1 and ICAM-1 in group I ranged from 5,700 - 16,500 pg/ml and from 210 to 1580 ng/ml respectively with a mean of 9971 ± 2554 pg/ml and 633 ± 331 ng/ml, while the serum levels of TGF-β1 and ICAM-1 in group II ranged from 1,570 -10,800 pg/ml and from 170 to 560 ng/ml respectively with a mean of 5708 ± 2331 pg/ml and 339 ± 108 ng/ml. Using ROC analysis, a cut off of TGF-β1 of 7210 pg/ml was found to

discriminate HCC- related HCV patients from non HCC related HCV patients with sensitivity 93.3 % and specificity 80%, while a cut off of ICAM-1 of 445 ng/ml could discriminate HCC- related HCV patients from non HCC related HCV patients with sensitivity 73.3% and specificity 83.3% (figure 2). Comparative statistics of serum levels of TGF-β1 and ICAM-1 are presented in (tables 1 and 2)

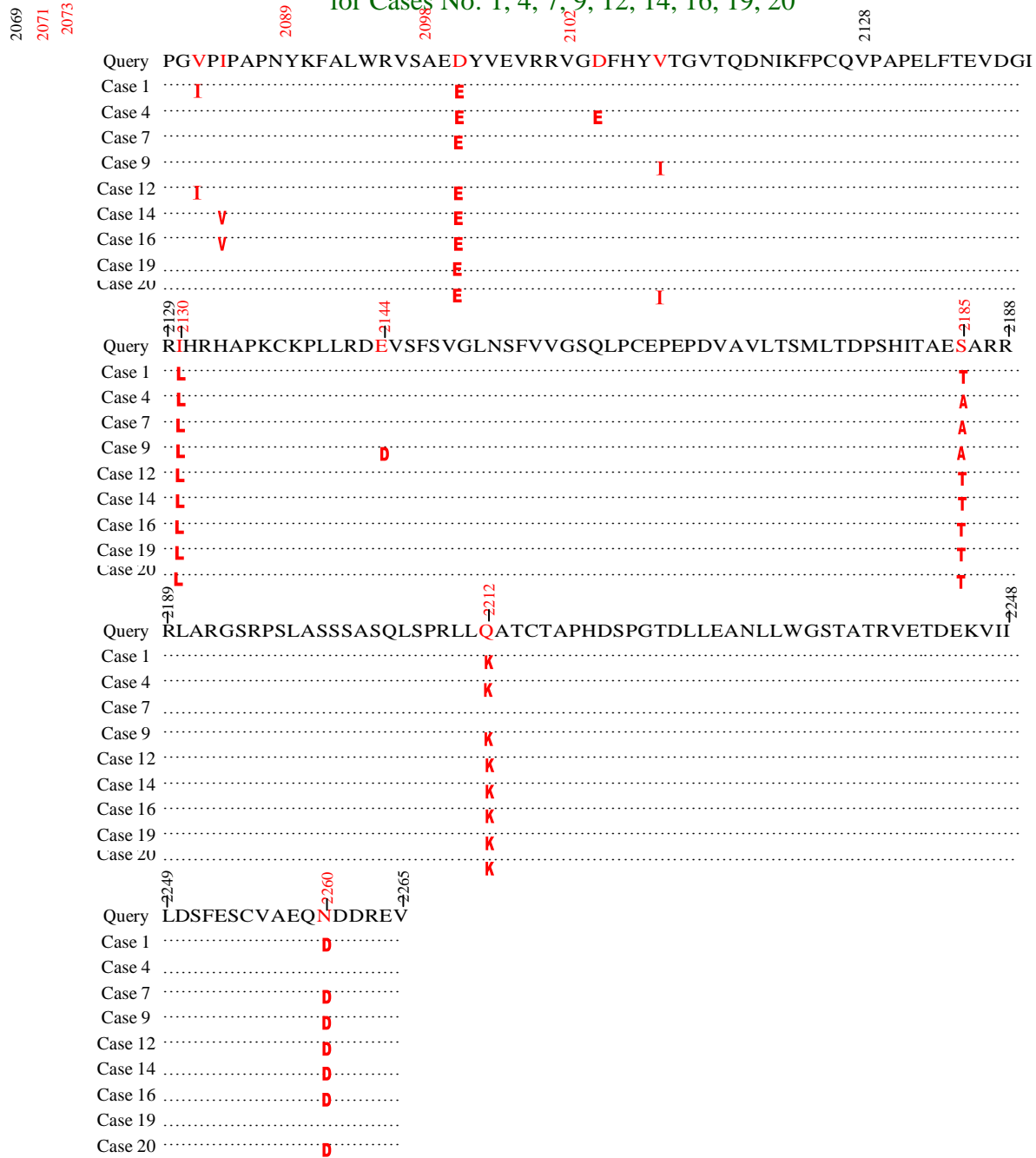
Logistic Regression

The results of regression analysis showed that serum TGF-β1 levels, serum ICAM-1 levels, and number of NS5A mutations are the significant variables for the diagnosis of HCV-related HCC ($p=0.004$, $p=0.02$, and $p=0.01$ respectively).

Table.1 Demographic and Descriptive Laboratory Data of Group I and Group II Patients

Parameter	Group I	Group II
Age (years)	≤ 55: 11 (36.7%) >55: 19 (63.3%)	≤ 55: 16 (53.3 %) >55: 14 (46.7 %)
Sex	Male: 27 (90%) Female: 3 (10%)	Male: 24 (80%) Female: 6 (20%)
HCV viral load (IU/ml)	Range: 2800-6,20000	Range: 1200-5,45000
NS5A sequence	wt: 18 (60%) 1-6 mutations: 12 (40%) >6mutations: 0 (0%) Range: 0- 6	wt: 7 (23.3%) 1-6mutations: 5(16.7%) >6 mutations: 18 (60%) Range: 0-17
ISDR sequence	wt: 20 (66.7%) 1mutation: 10 (33.3%) ≥ 2mutations: 0 (0%) Range: 0-1	wt: 7 (23.3%) 1mutation: 2 (6.7%) ≥2mutations: 21 (70%) Range: 0-7
TGF-β1 (pg/ml)	Range: 5,700 -16,500	Range: 1,570 -10,800
ICAM-1 (ng/ml)	Range: 210-1580	Range: 170-560

Figure.1 Schematic Representation of the Amino Acids Sequence of the Amplified Ns5a Region for Cases No: 1, 4, 7, 9, 12, 14, 16, 19, 20



Query : wild type NS5A genotype 4a from gene bank

Table.2 Comparative Statistics between Group I and Group II Patients as Regards the Studied Parameters

	Group I (n=30) Mean ± S.D	Group II (n=30) Mean ± S.D	t	p	Significance
Number of NS5A mutations/case	1.97 ± 0.47*	6.60 ± 5.06	-4.679	< 0.001	HS**
Sequence identities(%)	96.6 ± 3.31	91.6 ± 5.76	-3.99	< 0.001	HS**
Number of ISDR mutations/case	0.33 ± 0.087*	2.33 ± 1.79	-5.222	< 0.001	HS**
TGF-β1 (pg/ml)	9971 ± 2554	5708 ± 2331	7.009	< 0.001	HS**
ICAM-1 (ng/ml)	633 ± 331	339 ± 108	4.626	< 0.001	HS**

*Std Error of Mean (SEM) ** HS: highly significant

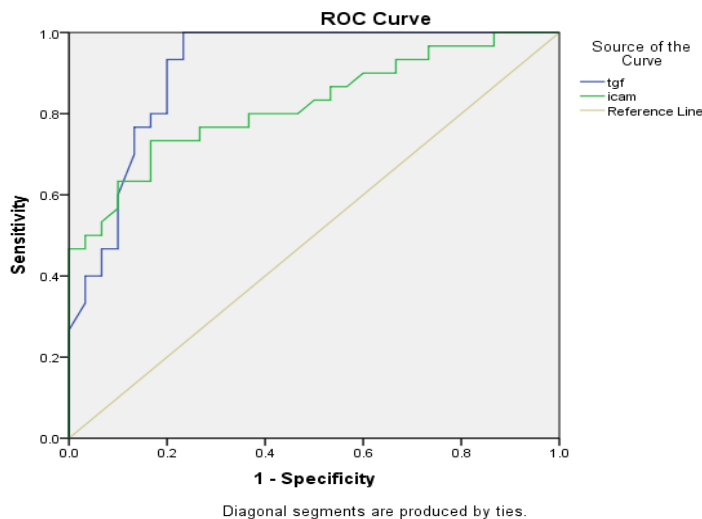
Table.3 Comparative Statistics between Group I and Group II Patients Who had Ns5a Mutations as Regards the Studied Parameters

	Group I (n=12) Mean ± S.D	Group II (n=23) Mean ± S.D	t	p	Significance
Number of NS5A mutations/case	4.5 ± 1.38	9.5 ± 3.01	-3.90	0.004	S*
Sequence identities (%)	91.5 ± 1.31	89.1 ± 3.82	-2.21	0.054	NS**
Number of ISDR mutations/case	1.0 ± 0.0	2.97 ± 1.33	-3.631	0.008	S
TGF- β1 (pg/ml)	9576 ± 2028	5913 ± 2133	2.528	0.021	S
ICAM-1 (ng/ml)	770.3 ± 429	327 ± 105	2.794	0.032	S

*S: significant

**NS: non significant

Figure.2 ROC Analysis Showing Cutoff Value of TGF-β1 and ICAM-1 to Discriminate between HCV-related HCC and Non HCC Patients



Chronically infected HCV patients have increased risk of developing HCC, which is directly correlated with the degree of liver damage and cirrhosis. In fact, HCC rarely occurs in non cirrhotic HCV patients, while the annual incidence rate of HCC in cirrhotic HCV patients is about 1 - 7% (16). The development of markers for identification of HCV infected people who are at high risk of progressing to fibrosis and developing HCC is thus of ultimate importance.

The HCV NS5A protein is thought to play a critical role in viral replication and assembly, inhibition of response to interferon, and hepatocarcinogenesis, which makes NS5A an appealing target for novel anti-HCV therapies (12). The difference in the viral load, clinical sequence of HCV infection, progression to cirrhosis and HCC development can thus be attributed to genetic differences in the sequence of HCV NS5A region which varies in different HCV genotypes, and as far as we know, no such study has been performed on Egyptian patients. In that context, sequence analysis of the amplified NS5A region was performed in both HCV-related HCC patients and chronic HCV non-HCC patients in the present study to highlight the potential pathogenic role of NS5A in the development of HCV-related HCC. We demonstrated that 18 patients (60%) out of 30 patients of HCV-related HCC group (group I) harbored a wt sequence of NS5A region with sequence identities (100%), while only 7/30 of the non HCC chronic HCV patients group (23.3%) harbored a wt sequence of NS5A region with a significant difference between both groups ($p=0.003$), and that 23/30 patients within the non HCC group (group II) harbored mutations in NS5A region that ranged from 5-17 with an average 9.5 ± 3.01 mutations and mean sequence identities (89.1%), while the 12/30 patients of the HCC group (group I) harbored mutations

that ranged from 2 - 6 with an average 4.5 ± 1.38 mutations and sequence identities (91.5%). These results are in agreement with the results of other previous studies (17,18,19), who reported that a significant majority of HCC patients had wild-type or minimally mutated NS5A protein, without substantial differences between tumor, liver tissue and serum, and suggested that the wt NS5A protein would probably have a role in HCV-related liver carcinogenesis.

Experimental evidences suggest that HCV-NS5A protein with a wt ISDR sequence (Codons 2209-2248) may contribute to the response to interferon based therapies, uncontrolled cell proliferation, and malignant transformation (18,19). This is enforced by the findings in the present study, indicating that there was a significant increase in mean number of mutations in ISDR region ($p<0.001$) in group II compared to group I, and that fewer mutations in the NS5A- ISDR, and wild type amino acid in this region were significantly correlated with HCC. Similarly, Hung and his associates (19), who investigated the association of risk of HCC development with the variation in the amino acid sequence in the NS5A-ISDR region, found that HCC was significantly associated with fewer amino acid substitutions in the NS5A-ISDR region, and that those patients who had ≥ 4 mutations in NS5A-ISDR region had a lower prevalence of HCC than those patients with few (<4) or no mutations (wild type) in this region, and suggested that amino acid substitutions in ISDR is a significant factor associated with the development of HCC in chronic HCV-1b patients. Meanwhile in our study, we found that only 10/30 group I patients had mutations in the ISDR region and none of them had more than 1 ISDR mutation, while 23/30 group II patients had ISDR mutations ranging from 1 - 7 with mean 2.97 ± 1.33

with a significant difference between both groups, and we suggest that NS5A-ISDR mutations < 2 has a significant association with HCV-related HCC in Egyptian patients with genotype 4a.

Based on these results, it is evident that NS5A wt sequence would indeed increase the risk for HCV associated HCC and thus could be used as a marker to identify HCV patients who are at risk to develop HCC and thus should be subjected to therapy in early phases before development of cirrhosis as it was reported that achieving sustained virological response in presence of cirrhosis would not abolish the risk of developing HCC (20).

The mechanism by which wt NS5A increases the risk of HCC is not fully elucidated (21), however it may involve escape from apoptosis, which is a crucial event that favors HCV survival and promotes hepatocarcinogenesis, by inhibition of caspase-3, activation of cellular proliferative pathways as Raf/MAPK/ERP pathway, and inducing mitotic dysregulation and chromosomal instability (16,21).

In conclusion, the wild type NS5A, particularly that of ISDR regions, is a significant marker for the development of HCV-related HCC. In addition, high levels TGF- β 1 and ICAM-1 are potential effective serological markers for the diagnosis of patients with HCV-related HCC.

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